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In vitro Antileishmanial Activity of Extracts from Endemic Moroccan Medicinal Plant *Salvia verbenaca* (L.) Briq. ssp *verbenaca* Maire (*S. clandestina* Batt. non L)

Abdeslam Et-Touys[1,2], Hajiba Fellah[1], Faiza Sebti[1], Meryem Mniouil[1,3], M'hammed Aneb[2], Houria Elboury[2], Ahmed Talbaoui[2], Nadia Dakka[2], Abderrahim Sadak[3] and Youssef Bakri[2*]

[1]National Reference Laboratory of Leishmaniasis, National Institut of Health (INH), Rabat, Morocco.
[2]Laboratory of Biochemistry and Immunology, Department of Biology, Faculty of Science, Mohammed V University, Rabat, Morocco.
[3]Laboratory of Zoology and General Biology, Department of Biology, Faculty of Science, Mohammed V University, Rabat, Morocco.

Authors' contributions

This work was carried out in collaboration between all authors. Study design was suggested by authors YB and AS followed by plant collection and extraction by author AET. Authors AET, MA and AT managed the literature searches. Authors AET, MM, FS, HE, ND and YB analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to evaluate *in vitro* the antileishmanial activities of organic extracts (methanol, *n*-hexane and dichloromethane extract) from *Salvia clandestina* (Lamiaceae) used in Moroccan medicinal plant.
Study Design: Evaluation of *in vitro* antileishmanial activity of extracts and determination phenolic contents.

**Corresponding author: E-mail: ybakri@gmail.com;*

Place and Duration of Study: After plant collection from the region of Rabat-Morocco, further work was carried out in Parasitology Laboratory of the National Institute of health and Laboratory of Biochemistry-Immunology, Faculty of Science, Mohammed V University of Rabat, Morocco from February 2015 to march 2016.

Methodology: The plant was extracted using organic solvents and using Soxhlet. The antileishmanial activity of extracts was tested against three leishmanial strains, *Leishmania major, Leishmania tropica* and *Leishmania infantum* in their promatigotes form, using MTT assay. The total phenolic content was assessed by the Folin-Ciocalteau assay and total flavonoid content was assessed by aluminium chloride ($AlCl_3$) colorimetric assay.

Results: The MTT based colorimetric assay showed reduced promastigotes viability on the all strains tested. The best growth inhibition was observed with *n*-hexane and dichloromethane extracts of *Salvia clandestina* (IC_{50}≤ 155.43 µg/ml) compared to N-methyl glucamine antimoniate (Glucantime®) (IC_{50}>1000µg/ml) used as control, after 72 h of treatment. Phenolic content of *S. clandestina* extracts ranged between 107.52±3.12 and 74.41±4.96 mg GAE/g extract, and the flavonoid content ranged between 24.64±3.65 and 16.31±3.69 mg QE/g extract.

Conclusion: The current investigation reveals that *S. clandestina* extracts possess activity against three Leishmania species. *S. clandestina* need further investigation so that the pure bioactive antileishmanial compounds should be isolated with cost effective, promising results and less side effects.

Keywords: Leishmania major; Leishmania tropica; Leishmania infantum; antileishmanial activity; Salvia clandestina.

1. INTRODUCTION

Leishmaniasis, parasitic disease caused by *Leishmania* sp genus, continues to present a major cause of mortality in the world. Based on worldwide estimate, in 2012, about 12 million people are diagnosed with leishmaniasis with high endemicity in developing countries [1]. In South East Morocco, this disease causes high morbidity and mortality rates [2,3,4]. The most common manifestations of this infection are represented by cutaneous leishmaniasis caused by *L. major* and visceral leishmaniasis caused by *L. infantum*. The conventional antileishmanial treatments used nowadays is based on synthetic drugs such as amphotericin B [5]. However, this treatment is expensive and causes many side effects toxicity and present limited antileishmanial activity [6]. Despite extensive research on treatment modalities towards *Leishmania* species, it has being a worldwide problem. In recent times, there have been increases in antileishmanial resistant strains of clinically important parasites. Thus, there is an urgent need for a permanent search and development of new drugs. Much attention has recently been paid to the discovery and development of new, more selective antileishmanial agents. Natural products with biological activity have received much interest over the past few years. Among the potential sources of new agents, plants have long been investigated. They contain large number of bioactive compounds that can be of interest in therapeutic. A variety of essential oils has been shown to possess antibacterial, anti-viral, antiparasitic properties and antileishmanial therapy [7,8,9]. Morocco is one of the developing countries with enormous diversity of plants. In Morocco, the use of traditional medicine is widespread practice. About 70% of the population uses traditional medicine, mainly from plants [10,11,12]. Previous studies conducted in our laboratory, demonstrated the antimicrobial and cytotoxic activities of many Moroccan medicinal plants [13-17]. *Salvia clandestina* has been used in traditional medicine in Morocco [12,18,19]. The plant studied here is an important medicinal plant, widely grows in most parts of Morocco and has an extensive application possibility in the traditional medicine against Leishmaniasis. However, there are few reports focusing on its antileishmanial effects. It is used against infections presented by cutaneous leishmaniasis [20]. To the best of our knowledge, no previous study of the antileishmanial activity of *S. clandestina* extracts has been reported. We perfomed *In vitro* antileishmanial assays on *L. major, L. tropica and L. infantum* promastigotes form. There are two forms of the parasite: The no mobile amastigotes form, which is intracellular and the flagellate extracellular promastigotes form [21]. Our study is conducted on the promastigotes form.

2. MATERIALS AND METHODS

2.1 Plant Material and Preparation of Extracts

Plant material was collected from Morocco, especially from Rabat (Northwest of Morocco). It was authenticated by Pr. Fatima Ezzahra EL ALAOUI-FARIS (Faculty of Sciences Rabat, Morocco). Whole plant part was dried at room temperature. The powdered materials were then weighed (200 g), and were sequentially extracted with *n*-hexane (1.2 L), dichloromethane (1.2 L), methanol (1.2 L) using Soxhlet, and filtered. The filtrate obtained was concentrated in a rotary evaporator (Heidolph Typ VV 1- Germany) to obtain the crude extract. The crude extracts were kept at 4°C until further uses.

2.2 Determination of Total Phenolic Content (TPC)

The concentration of the phenolic compounds in the plants extracts was determined using the Folin Ciocalteu assay [22], with some modifications. In brief, the extract was diluted to the concentration of 1 mg/ml, and aliquots of 100 µl or a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l) were mixed with 500 µl of Folin Ciocalteu reagent (previously diluted 10-fold with distilled water) and 400 µl of Na_2CO_3 (7%). After 40 min of incubation at room temperature (23±2°C), the absorbance was measured at 760 nm using a Spectrophotometer (Stat Fax- 2100) against a blank sample [23]. The total phenolic content was calculated using a calibration curve for gallic acid (R^2= 0.998). The results were expressed as the gallic acid equivalent per gram of dry weight of extract (mg of GAE/g of extract). Alls samples were analyzed in triplicate.

2.3 Determination of Total Flavonoid Content (TFC)

The total flavonoid content of the extracts was determined using the aluminum chloride ($AlCl_3$) colorimetric method described by Brighente et al. [24] with minor modifications. Briefly, 1 ml of the extract (1 mg/ ml in methanol) or a standard solution of quercetin (20, 40, 60, 80 and 100 mg/l) were mixed with 1 ml of 2% $AlCl_3$ in methanol. After 40 min of staying at room temperature (23±2°C), The absorbance against blank was measured at 430 nm using a Spectrophotometer [23]. The total flavonoid content was calculated using a calibration curve for quercetin (R^2= 0.985). The results were expressed as the quercetin equivalent per gram of dry weight of extract (mg of QE/g of extract). All samples were analyzed in triplicate.

2.4 Cell Viability Assays

The *In vitro* antileishmanial effect of the extract obtained was evaluated on culture of three *Leishmania* species: *Leishmania infantum* (MHOM/MA/1998/LVTA), *Leishmania tropica* (MHOM/MA/2010/LCTIOK-4) and *Leishmania major* (MHOM/MA/2009/LCER19-09). The promastigotes form were isolated and identified in National Reference Laboratory of Leishmaniasis, National Institute of Health, Rabat-Morocco.

Parasites cultures of each *Leishmania* species were washed with phosphate buffered saline (PBS) and centrifuged at 1500 rpm for 10 min. Cells were then re-suspended in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum and 1% Penicillin-Spreptomycin mixture. Cultures were maintained at 23°C. The effect of the isolated extracts on cell viability was assessed using the 3-(4.5-dimethylthiazol-2-yl)-2.5 diphenyl tetrazolium bromid (MTT) assay, which measures the metabolic activity of mitochondria [25]. MTT assays are presently the prefered methods of cytotoxicity assessment in our laboratory [14,15,26]. The tests were conducted on 96-well microplates. Before treatment with extracts, 100 µl medium RPMI (GIBCO) containing $2.5x10^6$ promastigotes/ml were placed in each well containing RPMI (GIBCO) and cultured at 23°C for 72 h. After incubation, samples were treated with crude extracts. Exactly from the stock solution (10 mg/ml), each extract sample was applied in a series of 6 dilutions (final concentrations ranging from 1 µg/ml to 1000 µg/ml) in Dimethyl sulfoxid (DMSO 1%). Test solution (100 µl), was added in decreasing concentrations in duplicate. Microplates were then incubated for 72 h at 23°C. After, 10 µl MTT solution (5 mg/ml) (SIGMA) was added to the wells containing samples and were incubated for 3 h at 23°C. Tetrazolium salts are cleaved to formazan dye by cellular enzyme mitochondrial succinate dehydrogenase (only in the viable promatigotes). A solubilization solution (Isopropanol/hydrochloric acid) is added to dissolve the insoluble purple formazan product into coloration solution. The absorbance was measured at 570 nm, using microplate reader (Statfax 2100) [25]. Data are presented as

means ± SD of six different assays. Statistical analysis was performed by Origin 6.0 software.

2.5 Data Analysis

The measurements of total phenolic compounds, total flavonoids and antileishmanial activity were carried out for three replicates. The results are expressed as mean ± SD. The relationship between phenol contents and antileishmanial capacities was established using coefficient correlation.

3. RESULTS AND DISCUSSION

3.1 Phenol and Flavonoids Content

Total phenol content was estimated by the Folin-Ciocalteu colorimetric method in comparison with standard gallic acid and the results were expressed in terms of mg GAE/g dry extract using an equation obtained from a calibration curve. Organic extracts (Methanol, n-hexane and dichloromethane) of *S. clandestina* had an important charge of phenols and their values varied widely for solvent extract to another (Table 1).

The methanol extract had an importance value of phenol content (107.52±3.12 GAE mg/g extract), flowed by n-hexane and dichloromethane extract by 95.34±2.35 and 74.41±4.96 GAE mg/g extract respectively. Ours extracts must be considered as a very good source of phenolic compounds. Indeed, Tawaha et al. [27] have reported that if the phenolic content is higher than 20 mg GAE/g extract, it could be considered as very high. Phenolic compounds are secondary metabolites of medicinal plants and can exhibit antimicrobial activities [28]. These activities could be attributed to the hydrophobic character of phenolic content.

Flavonoids content was estimated by a colorimetric method using quercetin as standard flavonoid. Results were expressed in terms of mg QE/g dry extract using an equation obtained from a calibration curve. The concentration of flavonoids in the extracts is varied depending to the solvent of extraction. Their values ranging from 24.64±3.65 to 16.31±3.69 quercetin equivalents of extract (QE mg/g extract) (Table 1). *N*-hexane and methanol extract showed higher flavonoids content than dichloromethane extract. Flavonoids, a large group of polyphenolic compounds, have demonstrated several biological activities [23].

3.2 Cytotoxicity Effect

The investigation of the cytotoxic potential of extracts from *S. clandestina* Moroccan plant that is used in traditional medicine for treatment of various diseases was conducted on three *Leishmania* species: *L. major*, *L. tropica* and *L. infantum.* The present study was undertaken to provide comparative data on the *In vitro* antileishmanial activity of different extracts of Moroccan medicinal plant *S. clandestina.* Promastigotes were exposed to increasing concentrations ranging from 1 µg/ml to 1000 µg/ml. Assay by the MTT assay as described above, indicates that the extracts revealed different cytotoxic activities towards the three promastigotes species.

As shown in Fig. 1, Fig. 2 and Table 1, the *n*-hexane and the dichloromethane extracts of *S. clandestina* present important inhibiting effects against *L. major* and *L. tropica* IC_{50} values 155.43 µg/ml, 24.56 µg/ml, 148.23 µg/ml and 33.77 µg/ml respectively. Furthermore, the *n*-hexane extract showed high inhibitory effect on the *L. infantum* promastigotes growth (IC_{50} values 14.11 µg/ml) (Fig. 3). Whereas, the methanol extracts of *S. clandestina* presents less important inhibiting effects on the promastigotes growth with IC_{50} about of 1000 µg/ml for *L. major* (Fig. 1) and *L. infantum* (Fig. 3). For *L. tropica*, the IC_{50} was approximatively about of 850 µg/ml. On the other hand, less inhibitory effect on promastigotes was observed

Table 1. Total phenolic content (TPC) and total flavonoid content (TFC) of *S. clandestina* extracts

	Extracts		
	MeOH	**Dichloromethane**	***n*-hexane**
TPC (mg GAE[a]/g extract)	107.52±3.12	74.41±4.96	95.34±2.35
TFC (mg QE[b]/g extract)	23.60±1.42	16.31±3.69	24.64±3.65

TPC and TFC values are mean±standard deviation of three separate experiments.
[a]Gallic acid equivalents and [b]Quercetin equivalents.
MeOH: Methanol extract.

Table 2. The inhibitory concentration (IC_{50}) values in µg/ml from *Salvia clandestina* towards *Leishmania major*, *Leishmania tropica* and *Leishmania infantum* promastigotes is determined by the MTT assay

Extracts	*Leishmania major*	*Leishmania tropica*	*Leishmania infantum*
n-hexane	155,43	148,23	14,11
Dichloromethane	24,56	33,77	31,57
MeOH	>1000	850,76	>1000
Glucantime®	>1000	>1000	>1000

at concentration of Glucantime® used as reference. Methanol extract exhibited comparable IC_{50} values to the commercial drug Glucantime®, the concentrations providing 50% of inhibition (IC_{50}) values were about 1000µg/ml. Furthermore, dichloromethnol extracts from *S. clandestina* were found to be more active against the three promastigotes *Leishmania* species. Interestingly, we report here that the differential antileishmanial effects of these extracts was related not only to their chemical composition but also to the nature of the promastigotes species and the differential antileishmanial activity of these extract against the same promastigotes species is related to the differential composition of such extracts. Our results agree with previous research in which the best antileishmanial activity was observed with the dichloromethane extract of *Acanthospermum hispidum* and methanol extracts of *Calendula officinalis*, *Datura stramonium* and *Salvia officinalis* [29,30]. On the other hand, the mechanism of action of plant extract is still obscure. Specific cellular targets of our extracts studied here can be related to cell membrane disruption for their lipophilic properties and lead to cell lysis. Our most active extracts can also interact with mitochondrial membranes leading to its death by apoptosis. The results suggest that the high biological activities of the *n*-hexane and dichloromethane extract of the *S. clandestina* plants may be related to major compounds. This does not exclude the possibility that the other constituents may account for the biological property of the extracts. The synergistic effects of active chemicals of the extracts should be taken into consideration. The mechanism of action of extracts is not full understood but it is thought to involve walls and membrane disruption by the lipophilic compounds [7,31]. It is then important to develop a better understanding of their mechanisms of biological activity. In this scenario, the medicinal plant studied in our laboratory may be looked as an important source of new antileishmanial agents and opens a new file of investigation to discover mechanisms responsible for the observed activity.

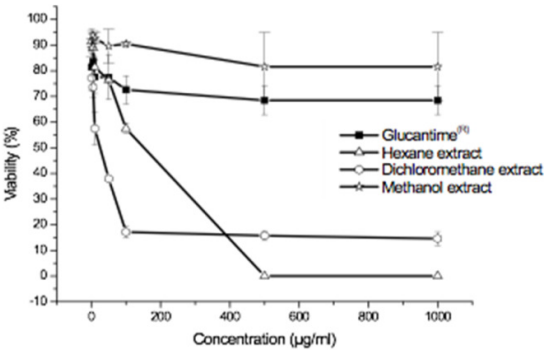

Fig. 1. Antileishmanial activity of extracts from *Salvia clandestina* plants against *Leishmania major* promastigotes. Promastigotes were incubated with different concentration of the plant extracts ranged from 1 µg/ml to 1000 µg/ml) for 72 h. Cell viability was determined by the MTT assay (n=6). Glucantime® was used as positive control. Data are expressed as means ± SD of six determinations tests Viability curves: Percentage viability =Absorbance of the test wells/Absorbance of control) x 100

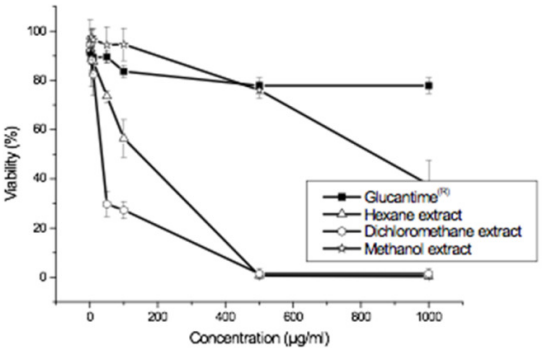

Fig. 2. Antileishmanial activity of extracts from *Salvia clandestina* plants against *Leishmania tropica* promastigotes. Promastigotes were incubated with different concentration of the plant extracts ranged from 1 µg/ml to 1000 µg/ml) for 72 h. Cell viability was determined by the MTT assay (n=6). Viability curves: Percentage viability =Absorbance of the test wells/Absorbance of control) x 100

Table 3. Correlation coefficient between phenolic content and antileishmanial activities

Components	Correlation coefficient		
	Leishmania major	***Leishmania infantum***	***Leishmania tropica***
Polyphenols	R^2 = 0,725	R^2 = 0,594	R^2 = 0,731
Flavonoids	R^2 = 0,257	R^2 = 0,146	R^2 = 0,263

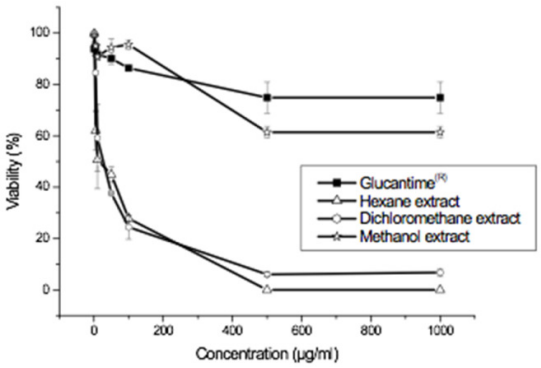

Fig. 3. Antileishmanial activity of extracts from *Salvia clandestina* plants against *Leishmania infantum* promastigotes. Promastigotes were incubated with different concentration of the plant extracts ranged from 1 µg/ml to 1000 µg/ml) for 72 h. Cell viability was determined by the MTT assay (n=6). Viability curves: Percentage viability =Absorbance of the test wells/Absorbance of control) x 100

3.3 Correlation Coefficient between Phenolic Content and Antileishmanial Activities

Relationships between the levels of phenols and flavonoids content of *S. clandestina* extracts and antileishmanial activities (IC_{50}) were established (Table 3). The correlation coefficient between total phenol content and *antileishmanial capacities* (IC_{50}) *was* R^2 = 0,725, R^2 = 0,594 and R^2 = 0,731 for *L. major, L. infantum and L. tropica respectively*. *While,* correlation coefficient between flavonoids content and antileishmanial capacities was R^2 = 0,257, R^2 = 0,146 and R^2 = 0,263 for *major, L. infantum and L. tropica.* These results showed that antileishmanial activity correlates moderately with total phenolic content. While, there is no correlation between this activity and flavonoids content. This suggests that there many other phenolic compounds, such as tannins, comarins etc… implicated in these activities.

4. CONCLUSION

The results obtained in this study indicate that the *n*-hexane extract and the dichloromethane extract of the plant *S. clandestina* were shown to induce significant and dose-dependent inhibitory activities against promastigotes of *L. major, L. tropica* and *L. infantum* promastigotes strains. These extracts were found to be more active against the chosen pathogenic *Leishmania* strains.

This study provides an important basis for further investigation into the isolation, characterization and mechanism of biological compounds. Thus, these plants could be as source for new lead structures in drug design and may be used together with known drugs in the development of pharmacological agents to combat leishmaniasis infectious diseases. Finally, Morocco possesses variety of plant species that might be important sources to treat different diseases.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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